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Conservation, Disease-indexing and Utilization of Pathogen-free Citrus and Banana Genetic Resources in Taiwan

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Abstract

Virus and virus-like diseases (greening) of citrus and banana have been causing considerable damage to fruit production worldwide due to their systemic invasion and common spread through infected budwoods, vegetatively propagated seedlings, and transmission by insect vectors. The heat-tolerant form of greening locally known as Huanglungbin (HLB) in China has seriously affected citrus trees in the tropical/ subtropical regions of Asia, and recently in the North and South America. The HLB caused great damage to the citrus industry by shortening tree lifespan and lowering fruit quality. Citrus tristeza virus (CTV) is the most common and destructive virus in the western hemisphere, and has a worldwide distribution. Citrus tatter leaf capillovirus (CTLV) has a potential to become prevalent when trifoliate orange and its hybrids are used as rootstocks. The citrus exocortis viroid (CEV) occurs in many countries, and its commercial effects are primarily on trees grafted on trifoliate orange and some its hybrids, and Rangpur lime. The bunchy-top virus (BBTV) disease has been the common and destructive virus disease of banana since the beginning of this century in Asian and Pacific regions. The banana mosaic caused by CMV has become epidemic after common cultivation of tissue-culture plantlets. Banana streak badnavirus and bract mosaic potyvirus have newly spread to many banana areas in the Pacific regions in recent years. These systemic diseases have become one of the serious constraints for the industry of citrus and banana in the Asian and Pacific region.

These diseases are generally controlled by the integrated control measures including cultivation of pathogen-free seedlings, elimination of inoculum sources and prevention of secondary spread by vector insects. In view point of germplasm utilization, establishment of pathogen-free nursery system is primarily important for preventing prevalence of these diseases. National scheme of citrus pathogen-free nursery system is consist of PF citrus

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foundation in Chia-Yi Agriculture Experimental Station repository and the approved nurseries in private sector and fruit cooperative. The production and utilization of PF-citrus seedlings has been performed under citrus sapling certificate regulation. The virus-free banana cultivars of economic importance obtained through virus-indexing and tissue culture under heat-therapy temperature have been kept in the foundation screen-house for mass production of PF banana TC-plantlets in Taiwan Banana Research Institute. The PF citrus and banana seedlings are commonly cultivated for improving the agro-industry in Taiwan. The precise and rapid indexing techniques are indispensable for management of pathogen-free nursery system and health management of citrus and banana plants in the field. Development and application of molecular diagnostic probes including monoclonal antibodies, DNA probes, and primer pairs for polymerase chain reaction (PCR) were made for formulating indexing techniques including ELISA, rapid diagnostic strips, PCR and RT-PCR to be discussed in this presentation . The establishment of virus-free nursery system for controlling citrus and banana virus diseases has been properly performed through shoot-tip micro-grafting, and tissue culture via virus-free stock indexing and heat-therapy with tissue culture in Taiwan.

Introduction

Citrus and Banana are the most important fruits for local consumption and export in Taiwan and the other Asian countries. Citrus production with so many cultivars, are the most important industries for fresh fruit consumption, and juice processing world-wide particularly in the tropic and subtropical areas. Banana and plantain (*Musa* spp) are the important crops for local consumption as fresh fruit and staple food, and export in the Asian, African and American countries. Some systemic virus and virus-like diseases have been causing considerable damage to fruit yield and quality, and have became one of the serious constraint for the production of citrus and banana world wild.

Citrus greening (Huanglungbin = HLB) was first reported in 1947 from South Africa. The psyllid borne virus-like disease caused by *Liberibacter asiaticus* has been devastation citrus industry in Taiwan since 1951. This disease has been prevalent in tropical and subtropical citrus areas in Asia and South Africa, and appeared in North and South America recently. This disease caused great damage to the citrus industry by shortening tree lifespan less than 10 years. Most citrus cultivars, except pummelo, were already susceptible to the Asian form of HLB before the 1970s. However, pummelo became infected by a new GO strain in Taiwan and Southeast Asia since 1971. **Citrus tristeza closterovirus (CTV)** was assumed originated in China quite a long time ago. The CTV caused a great damage to citrus industry in South America and Africa in the 1920s and has a worldwide distribution. Some new strains of CTV have evolved in South America and Asia in recent years, and have been causing severe

stem pitting, stunting and affecting fruit yield and quality. **Citrus tatter leaf capillovirus (CTLV)** was first found in 1962 in Mayer lemon introduced to California from China. Several lines of Satsuma mandarin grown in Japan have been found to be infected by CTLV since 1975 (Miyakawa, 1980). The virus has a potential to become prevalent when trifoliate orange and its hybrids are used as rootstocks. The CTLV has been commonly in most citrus cultivars without significant symptom in Taiwan. Exocortis is a bark-scaling and tree-stunting disease caused by **citrus exocortis viroid (CEV)**. The disease was first described as scaly butt in 1948 in Australia. The casual agent occurs in many countries, and its commercial effects are primarily on trees grafted on trifoliate orange and some its hybrids, and Rangpur lime. The CEV was scarcely found in sweet orange and Eureka lemon trees rooted on Rangpur lime in Taiwan. The greening disease commonly with mix-infection of citrus tristeza and tatter leaf viruses, causes sever yellowing and decline, and ultimately death of citrus trees.

Banana bunchy-top babuvirus disease has been the common and destructive virus disease of banana since the beginning of this century in Asian and Pacific regions. Several occurrence of outbreak become the limiting factor for banana industry in Taiwan during the past decades. **Banana mosaic** caused by **cucumber mosaic cucumovirus (CMV)** was first reported in New South Wales in 1929 and in Central American in 1957. This disease had become prominent in Taiwan in 1974, and severe outbreaks occurred recently after common cultivation of tissue-culture plantlets. **Banana streak badnavirus disease (BSV)** was first described in Mysore (AAB) from Ivory Coast in 1974. The BSV was found worldwide in most of the banana-growing areas in recent years, and had potential for spreading and becoming epidemic. There is the potential for serious yield losses with some virus isolates. Plant death was reported in Africa. Disease incidence varies between countries and this may be related to strain differences and vector activity. The virus infection was recently (1994) found in exotic Mysore and Latundan, and native wild banana in Taiwan. **Banana bract mosaic disease** was first noted in 1979 in Mindanao, the Philippines. It has subsequently been widespread throughout the Philippines. The disease has also been found in India, Sri Lanka, Vietnam, and Western Samoa. A potyvirus, **banana bract mosaic virus (BBrMV)** isolated from infected plants, has been assumed to be the causal agent. Yield losses of up to 40%in Cardaba (ABB) and Lakatan (AA) have been recorded in the Philippines. Particularly, the most important cultivar, Saba (ABB) is commonly infected. The virus has been found in dwarf Cavendish (AAA) seldom in Taiwan recently.

These systemic diseases are transmitted by vegetative propagation of seedlings such as sucker, cutting, tissue culture plantlets and bud-scion for grafting as well as insect transmission. These diseases are generally controlled by the integrated control measures including cultivation of pathogen-free seedlings, elimination of inoculum sources and prevention of secondary spread by vector insects. Establishment of pathogen-free nursery

system is primarily important for preventing prevalence of these diseases (Su and Chen, 1991). Shoot-tip micro-grafting (STG) of citrus and STG combined with heat therapy have been performed for obtaining virus-free foundation stock of citrus in Taiwan (Su and Chu, 1984). The virus-free nursery system for controlling banana virus diseases has been performed by tissue culture via with virus-free stocks indexing and heat-therapy with tissue culture in Taiwan. Pathogen-free citrus and banana germplasm foundation have been established for production and cultivation of pathogen-free citrus seedling and banana TC-plantlets.

The precise and rapid indexing techniques are indispensable for management of pathogen-free nursery system. Development and application of molecular diagnostic probes including monoclonal antibodies, DNA probes and primer pairs, were made for formulating indexing techniques (Tsai and Su, 1991; Hung, Wu and Su, 1999). As a rule, a virus disease and virus-like disease are diagnosed by characteristic symptoms and bioassay with indicator plants. Nevertheless, disease indexing and detection of the causal agents are precisely and rapidly performed by enzymelinked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and reverse-transcription (RT) PCR with molecular diagnostic probes.

Disease Indexing of Citrus Greening and Virus Diseases

1. Citrus greening pathogen is commonly detected by polymerase chain reaction (PCR) analysis

Detection of HLB in symptomless citrus plants is accomplished by PCR test which has been commonly applied to indexing citrus foundation stocks and pathogen-free seedlings (Su, Hung, Wu, 1995). The sensitivity of HLB detection is uplifted by development and application of PCR analysis with adequate primer pairs (F: CAC CGA AGA TAT GGA CAA CA; R: GAG GTT CTT GTG GTT TTT CTG) (Huang, Wu and Su, 1999). The PCR-amplified DNA products are analyzed by ordinary electrophoresis (Fig. 1). (Protocol referring to the following chapter on the detection of banana viruses).

2. Citrus tristeza virus is commonly detected effectively by ELISA with polyclone and monoclonal antibodies. Development and application of monoclonal antibodies through hybridoma technology was made for differentiation certain strains of CTV (Tsai and Su, 1991). Rapid diagnostic strip has developed with monoclonal antibody for CTV detection. The globulins labeled with colloidal gold, are used for preparing the strips. The virus antibodies-secondary anti-mouse antibodies are fixed in upper part of chromatographic paper strip. (ELISA protocol referring to the following chapter on the detection of banana viruses).

Protocol of diagnostic strips:

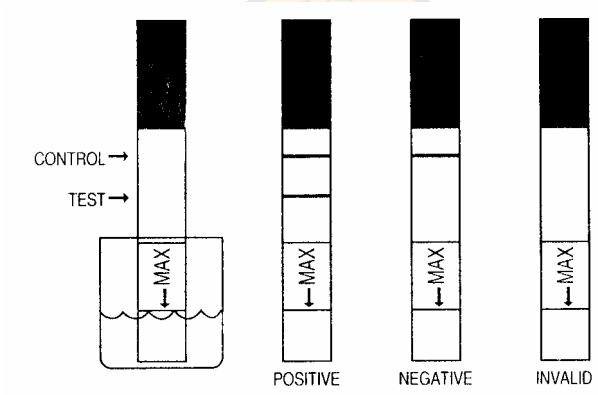
Collect 0.2~0.3 grams of tissue specimen from several leaves by cutting into pieces into an eppendorf tube.

(1) Grind the tissue pieces within 0.8ml of extraction buffer in an eppendorf tube by using bamboo/ wooden stick.

(2) Remove the test strip from its protective pouch (bring the test to room temperature before opening the pouch to avoid condensation of moisture on the membrane).

(3) Immerse a strip in the extraction buffer solution in eppendorf tube with arrow end pointing toward the solution not over labeled antibody pad. Do not immerse the strip over the MAX line.

(4) Wait for pink-colored bands to appear. Depending on the concentration of CMV, positive results may be observed within 2~3 minutes. However, to confirm negative results, the complete reaction time of 20 minutes is required.



Interpretation of results:

(1) POSITIVE: Two distinct pink-colored bands will appear, one in the plant test region and one in the control region.

(2) NEGATIVE: Only one pink-colored band appears in the control region. No apparent pink band appears in the plat test regions.

(3) INVALID: A total absence of pink-colored bands in both regions. If neither a test band nor a control band appears on the membrane, the test should be considered void. Improper testing procedures or deterioration of reagents probably occurred.

3. Citrus tatter leaf virus detection: ELISA with monoclonal antibodies has been developed, however its sensitivity of detecting the virus is low. Reverse transcription PCR (RT-PCR) was formulated by using cDNA primer pair (F: GGA AGA CTC ACA TAG ACC CG; R: TAC TCT

CCG AAC CTG CCT C) derived from the sequence of CTLV (Ohira *et. al*., 1995) for detecting the virus more rapidly. (RT-PCR protocol referring to the following chapter on the detection of BBrMV virus).

4. Citrus exocortis viroid is detected by RT-PCR with cDNA primer pair **(**F: GCT CCA CAT CCG ATC GTC; R: TGG ACG CCA GTG ATC CGC**).** (RT-PCR protocol referring to the following chapter on the detection of BBrMV virus).

Establishment of Pathogen-free Citrus Foundation Stock and Nursery System

In order to control the systemic virus and greening disease of citrus, the national scheme of virus-free nursery system was initiated, and conducted under joint program of R and D including NTU, TARI, and TBRI in Taiwan since 1983.

1. **Micro-grafting for Obtaining Pathogen-free Citrus Foundation Stocks.**

An improved method of shoot-tip micro-grafting (STG) was developed by using triangle-hole cut (Su and Chu, 1984) instead of inverted T cut described by Murashige *et al*. (1972). The STG program and health indexing were conducted in the Plant Virology Laboratory, National Taiwan University. The outline of STG schedule is as follows (Fig. 2):

Etiolated stock seedling grown on solid media
(Troyer citrange or other rootstock, 2 week-old in vitro)
↓
Micro-grafting shoot-tip into rectangular triangular hole,
Or V-cleavage of decapitated seedling
↓
Culture on liquid medium, incubate in the dark for 2 days.
↓
Growing in growth chamber or green house (28-30^{o}C)
Under cheese cloth shading for 2 weeks.
↓
Growing under uncovered condition for 2-3 weeks.
↓
Double grafting to potted rootstock seedlings
(Rough lemon, or other rootstock), and cultivate in green house
↓
The solid and liquid media were prepared following Murashige *et al.* (1972).

↓

※ Preparation of Citrus STG Medium (pH 5.7)

(1) Solid medium for growing STG seedling: MS salt mixture (Gibco BRL), 4g; Sucrose, 20g; ddH_2O, 1L; Agar, 1% (10g)

(2) Liquid medium for growing STG seedling: MS salts, 4.2g; Sucrose, 40g; growth factors (1L/100x stock: i-inositol, 100mg; thiamine-HCl, 0.2mg; pyridoxine-HCl, 1mg; nicotine acid, 1mg)

↓

2. National scheme of citrus pathogen-free nursery system

Pathogen-free citrus foundation stocks. The stocks are produced by means of improved method of shoot-tip grafting and heat-therapy in the Plant Virus Laboratory, National Taiwan University (PVL/NTU) and the Chia-Yi Agricultural Experimental Station of Taiwan Agricultural Research Institute (CAES/TARI) (Fig. 3). The STG-seedlings indexed as pathogen free by PVL/NTU, are used as foundation stock, and are transferred to the National Pathogen-free Citrus Foundation Stock Collection in screen-houses in CAES/TARI.

Scion-Propagation parent trees. Pathogen-free bud-woods are multiplied in scion-propagation trees derived from foundation stocks in insect-proof screen-house of CAES/TARI, the Taiwan Fruit Marketing Cooperative (TFMC) or approved nurserymen.

Pathogen-free citrus nurseries. Pathogen-free seedlings are produced by using pathogen-free scions derived from the parent trees in the Healthy Nursery Screen-houses operated by each branch office of the Taiwan Fruit Marketing Cooperative (TFMC) and approved nurserymen.

Citrus growers. The healthy certificated citrus seedlings are transplanted to the orchards, and protected by integrated control measures.

Note: The act of citrus bud-wood certificate has been established the production of pathogen-free citrus seedling in Taiwan since December of 2004.

3. Production of Pathogen-free Banana Seedling

These diseased might readily prevail if the mother stocks are infected latently with the causal viruses. Production of virus-free seedlings including suckers and tissue-culture (TC) plantlets through pathogen-free nursery system has to be managed by strict implementation of health indexing. The virus-free nursery system for controlling banana virus diseases has been properly performed through tissue culture via virus-free stock indexing and heat-therapy with tissue culture in Taiwan. The virus-free TC-seedlings of banana are widely grown for controlling banana virus in Taiwan.

Conservation of pathogen-free citrus germplasm

The citrus germplasm collection has been kept in Citrus gerplasm repository in Chia-Yi Agricultural Experiment Station, Agricultural Research Institute, Chia-Yi, Taiwan (Table 1).

Table 1. Citrus germplasm collection in Taiwan

Horticultural classification	Cultivars free from specific-pathogens	Cultivars not indexed
1. Mandarin		
Common mandarin	Bower, Dancy, Clementine, Encore, Esparta, Fremont, Genshokan, Guapile, Kara, Kinnow, Parson's special, Pixie, Ponkan, Early Ponkan, Sunburst, Sun Keim Woon	Drafted Ponkan
Satsuma	Okitsu No.3, Okitsu Wase, Miho Wase, Miyamoto, Nanka No.20, Emoto, Kowa, Nichinan-1	Yonezawa, Miyagawa Wase, Yamakawa, Kinho 1, Kinho 2, Kinho 3, Ohzu, Imamura, Aochima, Toman
Small fruit mandarin	Cleopatra, Depressa, Sunki,	Calamondin, Rangpur lime
2. Sweet orange		
Common	Fukuhara, Hong-Jian, Jaffa, Joppa, Liu-cheng, N. Hamlin, Suenaga, Parson Brown, Pera, Pineapple, Shamouti, Selectra, Sugar, Sun Wickom, White Siletta, Fukumatobeni	Seedless Liu-cheng, Mars early, Red Siletta, Tarocco, Trovita,
Valencia	Olinda, Cutter, Campell, Frost, Liu Gim Gong	Rhode Red
Blood	Moro, Sanguinella	
Navel	Dream, Frost, Morita, Ogawa, Fukumoto, Shirayanagi, Washington	Cara cara, Omishima, Yoshita
3. Tangor	Hai-Li, Iyo, King, Kiyomi, Murcott, Tankan, June-Tankan, Nova	Temple, Ortanique
4. Tangelo	Minneola	Orlando
5. Grapefruit	Duncan, O P Davis, Marsh, Frost Marsh, N. JBC 430 Marsh, Shamber, Star Ruby, Orablanco	Little River, Red Mexican, Thompson Pink, Red Blush
6. Pummelo	Kao Pan, MatoWentan, Thai White, Thaired pummelo,	Chandler, Mitsuyu, Red Wentan, Toyu, Wanpaiyu, Webber, Kaopan Yoshikawa Wentan,
7. Lemon	Eureka, Lisbon, Monachello,	Teresa, Villafranca
8. Lime	Bearss, Mexican	
9. Kumquat	Hong Kong, Oval, Meiwa,	Chang-shou
10. Citron	Buddda's hand, Etrog	
11. Rough lemon	Schaub	Milam, Common rough lemon

12. Sour orange	Australia, Chinotto, Common, Varigated sour, Bergamote	
13. Sweet lime	Palestine	
14. Trifoliate and hybrid	Common trifoliate, Rusk, Swingle, Troyer	Carrizo, Flying Dragon, Pomeroy
15. Miscellaneous	Excelsa, Meyer, Kawano, Shiranui, Natsudaidai, us 119	Beni-Hassaku, Excelsa, Hassaku, Hystrix, Hyuganaysu, Kimikan, Kinkoji, Konijimi, Kotokan, Kabuchi, Yuzu

*Germplasm free from greening pathogen, citrus tristeza virus and citrus tatter leaf virus.

The pathogen-free citrus cultivars obtained through shoot-tip micro-grafting and disease-indexing made in Virology Laboratory, Dept of Plant Pathology, NTU and CAES, Have been conserved in the Pathogen-free Citrus Foundation Screen-house of CAES. Pathogen-free citrus seedlings are produced by using the PF-scions supplied from the Foundation Center.

The pathogen-free nursery system has been properly performed through improved techniques of shoot-tip micro-grafting for obtaining virus/HLB-free citrus foundation stocks, and then production of healthy citrus seedlings under sapling certificate regulation in Taiwan.

Disease Indexing of Banana Virus Diseases

The reliable, sensitive and rapid indexing techniques are indispensable for management of pathogen-free nursery system and health management of banana plants in the field. Development and application of molecular diagnostic probes including monoclonal antibodies, DNA probes, and primer pairs for polymerase chain reaction (PCR) were made for formulating indexing techniques (Wu & Su, 1990; Su et al., 1997).

1. ELISA with monoclonal antibodies against BBTV and CMV.

It is difficult to purify a virus from virus-infected banana plant tissues due to high content of phenolic latex (Wu & Su, 1990; Wu et al., 1997). Therefore, it is better to produce monoclonal antibodies (McAb) of unique quality against banana viruses through hybridoma technique with impure virus preparation as immnogen. High specific monoclonal antibodies with high titer were prepared against BBTV and CMV respectively. Enzyme-linked immunosorbent assay (ELISA) with different McAbs was developed for virus detection and differentiation of the virus strains (Su *et al*., 2003). Adequate buffer solution has to be selected for virus extraction in ELISA test in order to eliminate interfering substances in banana latex

and stabilize virus extract. The adequate buffer for extracting BBTV is 0.5M Tris buffer (pH 7.5) containing 0.1% Na-DIECA, 5% sucrose and 0.5% skin milk, and for extracting CMV with 0.2M potassium-phosphate buffer (pH=7.4) containing 0.5% Na_2SO_3. Direct DAS-ELISA with alkaline phosphatase (AP)-conjugate of McAb (2H6) is used for BBTV detection, and CMV detection with AP-conjugate of McAb (7-1).

Protocol of virus detection by direct DAS-ELISA with AP-conjugate of McAb

(1) Micro-plates are coated with purified IgG of monoclonal antibody (2H6) against BBTV. Add 100ul of McAb (2ppm) in coating buffer to each well, and incubate the plates in a moist box at 37 °C for 2 hours.
(2) Wash the plates with PBS-T, 3~5 times.
(3) Add 100 µl/well of virus extracts from banana samples in virus extraction buffer at 1/5~1/20 dilution (W/V), then keep the micro-plates in a moist box at 37 °C for 2 hours or at 4 °C overnight.
(4) Wash the plates with PBS-T, 3~5 times.
(5) Add 100 µl/well of alkaline phosphatase (AP)-conjugate in PBS-T at 200x (0.5ppm) dilution. Incubate plates in a moist box at 37 °C for 2 hours.
(6) Wash the plates with PBS-T, 3~5 times.
(7) Add 100 µl/well of p-nitrophenyl phosphate (1mg/ml) in substrate buffer, and incubate plates in a moist box at 37 °C for 30~60 minutes.
(8) Read ELISA values of absorption at 405 nm.

Rapid Diagnostic Strips for BBTV and CMV

Monoclonal antibodies against banana bunchy-top virus (BBTV) and, cucumber mosaic virus (CMV) were developed by Laboratory of Plant Virology, National Taiwan University (e-mail: hjsu@ntu.edu.tw). The rapid diagnostic strips have been developed through cooperation by PVL of NUT and Meikang Biotechnology Company (e-mail: info@mei-kang.com). The globulins labeled with colloidal gold, are used for preparing the strips. The virus antibodies-secondary anti-mouse antibody are fixed in upper part of chromatographic paper strip (Protocol referring to the chapter on the detection of citrus virus).

Buffers：(Store at 4-6°C)

PBST (pH 7.4)	NaCl	8.0 g
	KH_2PO_4	0.2 g
	$Na_2HPO_4 \cdot 12H_2O$	2.9 g
	KCl	0.2 g
	NaN_3	0.2 g
	Tween 20	0.05 %
	dd H_2O	1 L
Coating buffer (pH 9.6)	Na_2CO_3	1.5 g
	$NaHCO_3$	2.93 g
	dd H_2O	1 L
Substrate buffer (pH 9.6)	Diethanol amine	97 ml
	H_2O	800 ml
	$MgCl_2$ $12H_2O$	100mg
	dd H_2O	1 L

2. Detection of banana bunchy-top virus (BBTV) and banana streak badnavirus (BSV) with polymerase chain reaction (PCR) followed by electrophoresis analysis

In order to increase sensitivity of virus detection and specificity of virus-strains differentiation, the primer pairs specific to BBTV strains were derived from the nucleotide sequences of BBTV-DNA through cloning and sequencing, and applied to formulation of the following protocol of PCR amplification followed by electrophoresis analysis. The primer pair specific to BSV quoted from J. Thomas' sequence, was applied to PCR analysis; the ordinary schedule for viral DNA/RNA extraction formulated through comparative trials, was commonly adapted for obtaining constant detection results. More recently, tissue-soak DNA extraction method mentioned below was developed for establishing rapid and inexpensive detection method of BBTV and BSV in PCR analysis.

PCR primer pairs:

※ **BBTV primer pairs:**

C1-CR:

F: 5'-GGA AGA AGC CTC TCA TCT GCT TCA GAG AGC-3'

R: 5'-CAG GCG CAC ACC TTG AGA AAC GAA AGG GAA-3'

S-CR:

F: 5'-GGG GCT TAT TAT TAC CCC CAG C-3'

R: 5'-AGC GCT TAC GTG GCG CAG CAC TAA CT-3'

SR-CR:

F: 5'-TGT CGT CGG CGA CGA AGT CG-3'

R: 5'-GGA CAT CCT CCT CCT TCA GAA GAG AGA-3'

※ **BSV primer pairs:**

F: 5'-CAA CTC AAG AGC CTA GTA TGC-3'

R: 5'-TAC CTC CGA CCG TAT TTC CAG-3'

PCR cycles for BBTV:

C-1 primer:

Step 1: 94 °C 4 min; 50 °C 1 min; 72 °C 2 min; 1 cycle.

Step 2: 94 °C 1 min; 50 °C 1 min; 72 °C 2 min; 30 cycles.

Step 3: 72 °C 10 min.

Step 4: 4 °C soaking.

S-CR, SR-CR primer:

Step 1: 94 °C 4 min; 60 °C 1 min; 72 °C 2 min; 1 cycle.

Step 2: 94 °C 1 min; 60 °C 1 min; 72 °C 2 min; 30 cycles.

Step 3: 72 °C 10 min.

Step 4: 4 °C soaking.

PCR cycles for BSV:

Step 1: 94 °C 4 min; 50 °C 1 min; 72 °C 2 min; 1 cycle.

Step 2: 94 °C 1 min; 50 °C 1 min; 72 °C 2 min; 30 cycles

Step 3: 72 °C 10 min.

Step 4: 4 °C soaking

Ordinary extraction of viral DNA (total nucleic acid):

1. Collect the leaf samples from banana plants infected with BBTV or BSV.
2. Froze the chopped tissue, 0.5g in liquid nitrogen and grind to fine powder in coffee blender or a mortar and pestle, or grind the fresh tissue directly.

3. Add 3 ml of DNA-extraction buffer* +1 % Sarkosyl (add 2.7 ml of DNA extraction buffer and 0.3 ml 10% Sarkosyl). Stir the tissue-powder suspension.
4. Transfer the suspension to a 1.5 ml eppendorf tube and incubate at 55 °C / 1hr in water bath.
5. Centrifuge the tube at 6,000 rpm / 5min.
6. Save sap supernatant (800 µl), add 100 µl of 5 M NaCl and 100 µl of CTAB / NaCl (10 % CTAB in 0.7 M NaCl), incubate at 65 °C / 10min.
7. Add equal volume of chloroform / isoamyl alcohol (24:1), mix thoroughly and spin at 11,000 rpm / 5 min (for BSV) or 6,000 rpm/ 5 min (for BBTV), save aqueous suspension.
8. Add equal volume of phenol / chloroform / isoamyl alcohol (25:24:1). Mix thoroughly and spin at 11,000 rpm / 5 min. (for BSV) or 6,000 rpm / 5 min (for BBTV), save aqueous suspension.
9. Add 0.6 volume of isopropanol to precipitate the nucleic acid. Incubate at -20 °C / 30 min. spin at 12,000 rpm / 20 min.
10. Wash pellet with 70% ethanol to remove CTAB residual. Briefly dry pellet and re-suspend in 100 µl of TE buffer. Store at -20 °C.

* DNA extraction buffer for BBTV and BSV in PCR analysis : 100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl, 100 µg / ml proteinase K (may be omitted); pH8.0 .

* DNA extraction reagents : 10% Sarkosyl (N-Lauroyl sarcosine); 5M NaCl; 10% CTAB (Hexadecryltrimethylammonium Bromide) in 0.5 M NaCl; chloroform: isoamyl alcohol (24:1); phenol: chloroform: isoamyl alcohol (25:24:1); isopropanol; 70% alcohol (ETOH); TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0).

3. Detection of banana bract mosaic potyvirus (BBrMV) by reverse-transcription PCR (RT-PCR) analysis

The formulated RT-PCR-electrophoresis analysis was applied to detection of BBrMV. The RT-PCR was developed by using the c-DNA primer pairs specific to BBrMV derived from B.C. Rodoni's sequence.

Extraction of viral RNA (total nucleic acids) from banana leaf tissues:

The present extraction procedure is almost identical with those for BBTV and BSV extraction.

cDNA primer pair for RT-PCR of BBrMV:

F: 5'-AAC GCT CAG CCT ACT TTT CG-3'

R: 5'-CAT ATC ACG CTT CAC ATC TTC A-3'

Thermocycles for RT-PCR of BBrMV:

Step 1: 50 °C 35 min; 94 °C 2 min.

Step 2: 94 °C 30 sec; 56 °C 30 sec; 68 °C 45 sec; 10 cycles.

Step 3: 94 °C 30 sec; 56 °C 30 sec; 68 °C 45 sec*; 25 cycles.

(*: 5 sec increasing ramp)

Step 4: 68 °C 7min.

Reaction mixture:

PCR reagents	**amount / tube**
ddH_2O	10µl
5x Superscript II buffer	2.5µl
10x Taq buffer	2.5µl
100 mM DTT	1.25µl
10 mM dNTPs	2µl
Taq polymerase (BRL or BerTaq)	0.2µl
Superscript II polymerase (BRL)	0.25µl
BBrMV primer pair (10 pmol /µl each)	1µl
RNA template	0.25µl

Establishment of Virus-free Banana-cultivar Foundation and Production of Pathogen-free TC-plantlets

The systemic diseases including virus diseases and Fusarial wilt might readily prevail if the mother stocks are infected latently with the causal viruses. Production of virus-free seedlings including suckers and tissue-culture (TC) plantlets through pathogen-free nursery

system has to be managed by strict implementation of health indexing. The pathogen-free (PF) banana cultivars of economic importance were obtained through virus-indexing and tissue culture under heat-therapy temperature (35 °C/3 months) (Wu and Su, 1991). The PF banana cultivars have been kept in the foundation screen-house of Taiwan Banana Research Institute (TBRI) (Fig. 4). The mass production of PF banana plantlets through tissue culture with the suckers from the foundation stocks (Fig. 6) has been properly performed in TBRI (Table 2).

Table 2. Pathogen-free foundation stocks in TBRI*

Genome type	Cultivars
AA	Kluai Khai
AAA	Taiwan Cavendish (Peichiao, Senrenchiao), Cavendish B. F (Africa), Grande Naine (Honduros), Lacatan (Jamaca), Poyo, Valery (Philippines), Vietnam Cavendish, Lacatan (Philippines), Gros Michel, William (Dwarf), Cavendish mutants resistant to Fusarial wilt (Formosana, Taichiao No. 1)
AAB	Latundan, Mas, Raja

*Cultivars free from BBTV, BSV, CMV, BBrMV, and Fusarial wilt pathogens

The virus-free banana TC-seedlings through hardening in screen-house 2 months (Fig. 7), are widely grown by farmers for controlling banana virus in Taiwan (Fig. 8).

Conservation of Banana Germplasm and Pathogen-free Banana Cultivars

The banana production was the most important agro-industry for local consumption and export during 1960~80s. Therefore, 242 cultivars of banana germplasms collection has been obtained from the domestic and foreign 34 banana-growing areas would-wild (Table 3). The cultivar collections have been kept within test tubes in germplasm bank (Fig. 5), screen-house and field conditions in Chia-Yi Agricultural Experiment Station, TARI, Chia-Yi, and TBRI, Pintung.

Banana streak badnavirus is commonly detection banana cultivars in symptom express form and virus-genome integrated form showing chloratic symptom or symptomless (Fig. 9). The cultivars introduced from INIBAP germplasm collection in Belgium, were found free from BBTV, CMV and BBrMV, but commonly infected by BSV of genome-integrated form (Table 4).

Table 3. Cultivar number of banana germplasm collection in TBRI (242 cultivars)

Genome type	Local *Musa* spp.			Foreign *Musa* spp.			Unknown origin		
	Test tube	Screen-house	Field	Test tube	Screen-house	Field	Test tube	Screen-house	Field
AAAA	0	0	0	10	10	6	0	0	0
AAAB	0	0	0	5	5	0	0	0	0
AABB	0	0	0	2	2	0	0	0	0
ABBB	0	0	0	1	1	0	0	0	0
AAA	11	11	7	87	87	70	4	4	4
AAB	1	1	0	27	27	20	16	16	16
ABB	5	5	2	14	14	9	8	8	8
BBB	0	0	0	4	4	2	0	0	0
AA	1	1	2	19	19	13	0	0	0
AB	0	0	0	2	2	2	0	0	0
BB	1	1	1	3	3	3	1	1	1
Abaca	2	2	2	4	4	4	0	0	0
Unknown	5	5	5	9	9	1	0	0	0
Total	26	26	19	187	187	130	29	29	29

Table 4. Virus detection of cultivars from Belgium (INIBAP)

	Cultivar name	Genome type	Virus detection			
			BBTV	CMV	BSV	BBrMV
I -1	Pisang Jari Buaya	AA	-	-	-	-
I -2	FHIA-01	AAAB	-	-	++	-
I -3	FHIA-02	AAAB	-	-	+	-
I -4	FHIA-03	AABB	-	-	+++	-
I -5	Williams (Bell, South Johnstone)	AAA	-	-	-	-
I -6	Cachaco	ABB	-	-	-	-
I -7	Gros-Michel	AAA	-	-	+++	-
I -8	Yangambi KM5	AAA	-	-	-	-
I -9	FHIA-17	AAAA	-	-	-	-
I -10	FHIA-23	AAAA	-	-	-	-

I -11	SH 3436-9	AAAA	-	-	-	-
I -12	TMB × 1378	ABBB	-	-	-	-
I -13	TMB × 5295-1	AAAB	-	-	+++	-
I -14	SH-3640	Hybrid	-	-	+++	-
I -15	FHIA 18	Hybrid	-	-	+++	-
I -16	FHIA 21 (#68)	AAAB	-	-	+++	-
I -17	CRBP 39		-	-	+++	-
I -18	FHIA-25	Hybrid	-	-	+	-
I -19	Pisang Ceylan	AAB	-	-	++	-

Discussion and Conclusion

(1) The production of citrus and banana fruit crops are the most important argoindustry world-wild for fresh-fruit consumption, staple food, processing and export as cash crops.

(2) Virus and virus-like diseases of citrus and banana have been causing commonly causing great damage to fruit production in fruit yield and quality due to their systemic invasion and epidemic spread through infected budwoods, vegetatively propagated seedlings, and transmission by insect vectors.

(3) In view of gremplasm utilization, establishment of pathogen-free nursery system is primarily important for preventing the prevalence of these systemic diseases.

(4) Development and application of precise and rapid indexing techniques are indispensable for management of pathogen-free nursery system and health management of PF seedling cultivation in the field.

(5) The conservation and effective utilization of citrus and banana genetic resources must be concerned to be free from the systemic virus, virus-like pathogens through disease indexing and pathogen elimination in germplasm (STG, and heat-therapy tissue culture).

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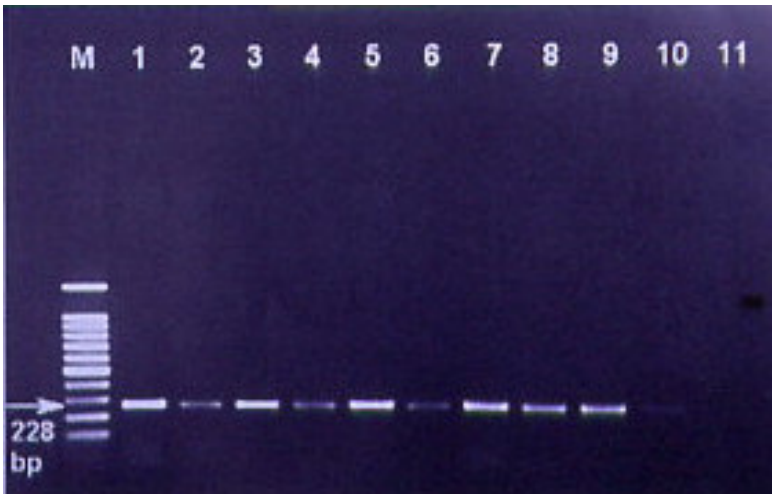
Figures



1~4: Taiwan GO isolates
5: Okinawa, Japan
6: China
7: Malaysia
8: Vietnam
9: Thailand
10: Saudi Arabia
11: Healthy CK

Fig. 1. Detection of HLB pathogen by polymerase chain reaction (PCR)/ electrophoresis analysis.

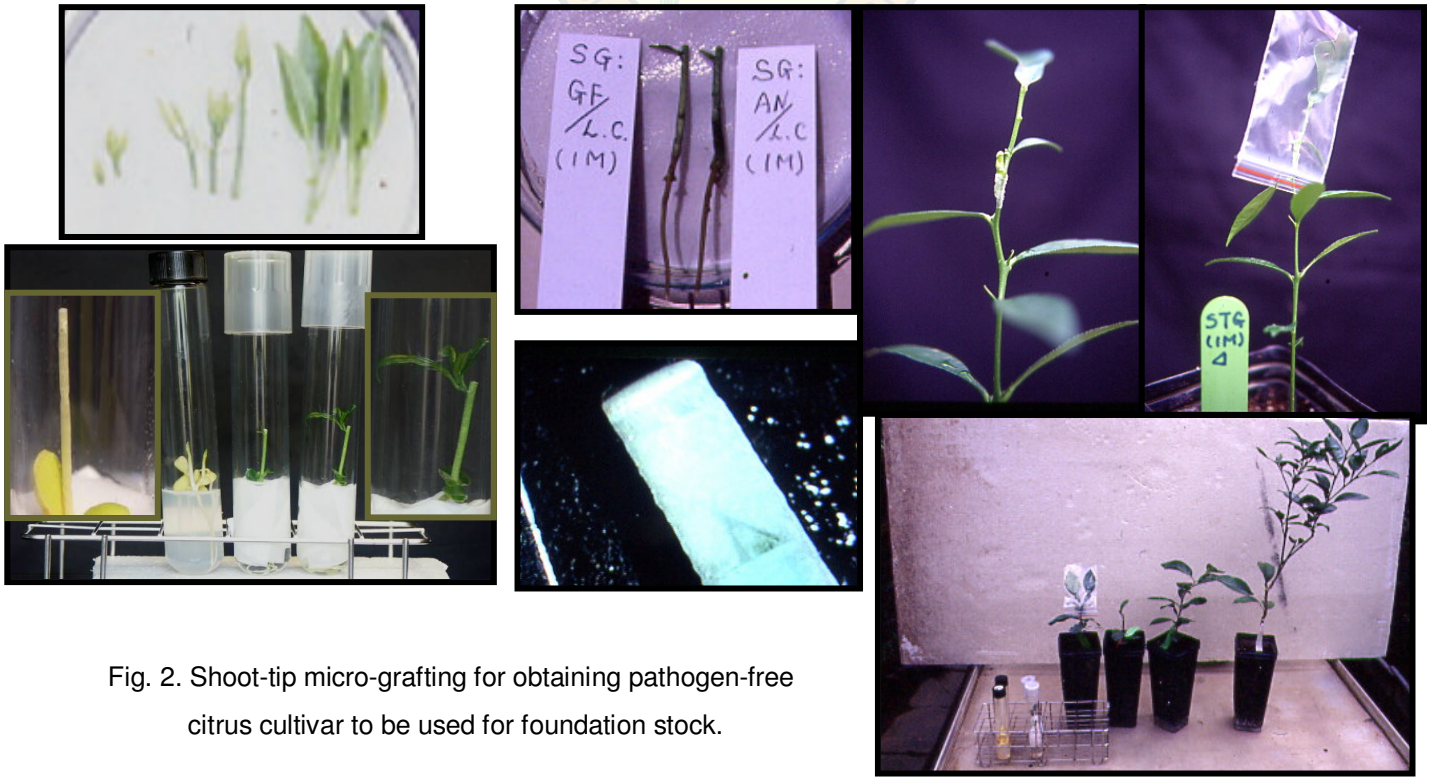


Fig. 2. Shoot-tip micro-grafting for obtaining pathogen-free citrus cultivar to be used for foundation stock.

Fig. 3. (Left) National Pathogen-free Citrus Foundation Stocks Collection Center, Chia-Yi Agricultural Experimental Station of Taiwan Agricultural Research Institute. (Right) Pathogen-free citrus foundation stocks kept in the insect-proof screen-house.

Fig. 4. Pathogen-free banana foundation stock in screen-house (TBRI)

Fig. 5. Banana germplasm collection in test tube kept at 17 °C (TBRI)

Fig. 6. (Left) Meristem culture from growing point of pathogen-free banana suckers
(Right) Multiplication of adventitious buds through tissue culture via 5~6 cuts.

Fig. 7. Mass production of PF banana TC-plantlets in tissue culture room (TBRI)

Fig. 8. Hardening of banana TC-plantlets in screen-house

Fig. 9. Banana plantation with PF TC plantlets

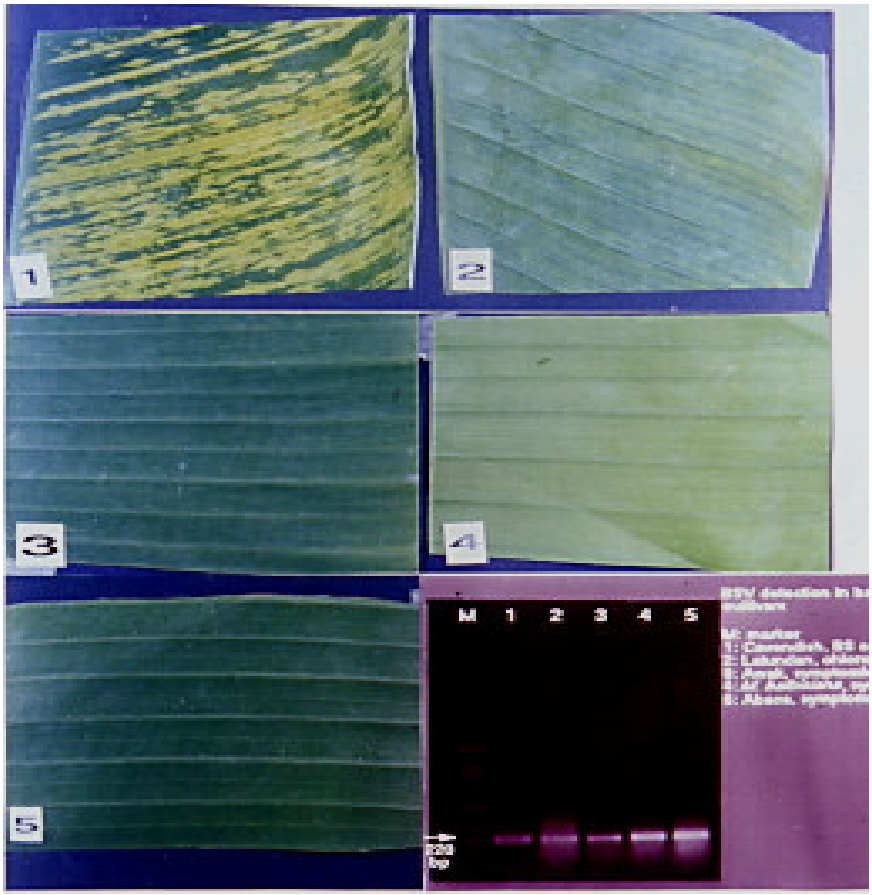


Fig. 10. BSV detection in various banana cultivars of different genome types by PCR test.

1. Cavendish (AAA)- BS severe; 2. Latundan (AAB)- chlorosis; 3. Awak (ABB)- symptomless

4. *M.balbisiana* (BB)- symptomless; 5. Abaca (*M. textilis*) - symptomless